

Methods in Pathology

Digital quantification of precursor frequency in the fallopian tube and its significance

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A high frequency of precursor lesions is a risk factor for cancer in many organ systems but must be precisely quantified. Pelvic serous neoplasia is associated with an estimated increase in frequency of secretory cell outgrowths (SCOUTs) with loss of PAX2 protein (PAX2p) expression (PAX2p-null SCOUTs) in the fallopian tube. However, to confirm this, PAX2p-null SCOUTs must be precisely quantified relative to the epithelial surface. We developed a method by which fallopian tube sections were digitized using an iScan brightfield scanner (BioImagene) and uploaded in Adobe Photoshop CS3 Extended. Pixel length was translated into microns and epithelial length measured with the Magic Wand tool. SCOUTs were expressed as a function of total epithelial perimeter. Frequency, required perimeter length, topographic clustering tendency and effects of age were ascertained. SCOUT frequency per 10 cm was 0–4.60 for cases and 0–1.66 for controls, averaging 0.84 and 0.27, respectively, ($P=0.007$). Required perimeter length for SCOUT detection was less in serous cancer cases and topographic distribution followed a random pattern without aberrant clustering. Age was also associated with SCOUT frequency ($P=0.025$) and differences between cancers and controls were still significant after adjusting for age ($P=0.001$). We describe an efficient method for quantifying epithelial perimeter in the fallopian tube and verify its relevance to precursor frequency. This has important implications for assessing precursor frequency both in the fallopian tube and in other organs—such as prostate, pancreas and colon—where epithelial precursors are integral to carcinogenesis.

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Although traditionally presumed to originate primarily in the ovarian cortex, high-grade ovarian cancers, specifically serous cancers, have been reassessed based on recent observations in the fallopian tube.¹ These include the frequent discovery of early serous cancers—termed tubal intraepithelial carcinomas—in the tubal fimbriae of women undergoing risk reducing salpingo-oophorectomy for germ-line *BRCA1* or *BRCA2* mutations, the observation of similar early cancers in 35–60% of un-selected women with fully developed pelvic

serous cancer, and the description of a precursor condition in benign tubal mucosa with altered TP53 expression, termed the 'p53 signature'.^{2–10} The latter has focused attention on the early molecular changes that may occur before the onset of either tubal intraepithelial or invasive serous carcinoma in the fallopian tube. Although the p53 signature, by virtue of its location (fimbria), p53 staining, evidence of DNA damage and proximity to intraepithelial carcinomas, is a presumptive direct precursor to intraepithelial carcinoma, a range of other discrete epithelial cell expansions have been described, termed secretory cell outgrowths or SCOUTs.^{11–13} SCOUTs share with p53 signatures a loss of PAX2 protein (PAX2p) expression but contain wild-type *TP53* and are not restricted to the distal fallopian tube. In recent reports, SCOUTs, specifically those lacking expression of the paired box 2 protein

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(PAX2p) have been found in greater frequency—per histologic cross-section—in fallopian tubes of women with serous cancer *vs* controls. These observations suggest that multiple events in the fallopian tube could be linked to serous carcinogenesis and that these events could be separated in space and time. If true, the notion of a single precursor sequence in a restricted location (fimbria) would be overly simplistic. However, resolving the importance of these disparate events requires attention to confounding variables such as age and equally important, the intrinsic sampling variation across different tissue sections, each with presumably different amounts of epithelium.¹⁴

Numerous examples in the literature attest to the importance of ascertaining precursor frequency or size as a surrogate for cancer risk, such as in the colon, pancreas and prostate.^{15–24} These organs house early epithelial changes that are not unlike the SCOUTs described in the fallopian tube. As high precursor frequencies could signal an acquired or underlying familial tendency for cancer development, precise quantification of the epithelial perimeter at risk is paramount. The purpose of this study was to devise a method of quantifying the surface area in the endosalpinx in histologic sections so that estimates of frequency of SCOUTs could be expressed in a more meaningful and reproducible algorithm. The implications pertain to accurately ascertaining whether the fallopian tube mucosa associated with serous cancers is unique by a greater susceptibility to clonal outgrowths of secretory cells. The potential significance of this approach in other tumor systems is discussed.

Materials and methods

Quantification of Fallopian Tube Precursor Frequency

This study was approved by the Human Investigation Committee at Brigham and Women's Hospital. Fallopian tube sections were selected from 34 patients with serous ovarian cancer and 35 controls. These have been previously used in a prior assessment of SCOUTs.¹² Each cross-section was immunostained with antibodies to PAX2p, as previously described and scored by two observers for linear segments of at least 30 consecutive non-ciliated cells exhibiting loss of PAX2p nuclear staining.^{11–13} For each case, a tally of PAX2p-null SCOUT number following examination of a single section from each block was made. To provide a denominator for the scoring, the epithelium from each tubal section was digitized and an objective epithelial length obtained using a 10-step model (Table 1; Figure 1, center). The fields were digitized using an iScan brightfield scanner (BioImage) and a scale bar was added. The images were then uploaded in Adobe Photoshop CS3 Extended. Pixel length was translated into microns and epithelial length measured using primarily the built in Magic Wand tool (Figures 1

Table 1 10-Step model for measuring oviductal epithelial perimeter

Step
1. Identify the epithelial lesions using H&E and/or immunoperoxidase stains
2. Digitalize the stained slides using a digital image scanner (eg, Bioimage iScan Coreo Au brightfield scanner)
3. Add a scale bar to the digital image
4. Take screenshots of the epithelial lining
5. Import the screenshots in Adobe Photoshop CS3 Extended (or a later version)
6. Use the Magic Wand tool to select the epithelial lining or the lumen in case of a confined space. Adjust the Magic Wand tolerance and/or image contrast/brightness as needed
7. Using the scale bar as a reference transpose pixels into microns (analyze→set measurement scale→custom)
8. Record measurements (analyze→record measurements)
9. The number in the 'Perimeter' column denotes the total epithelial length in microns
10. In case of measuring the epithelial lining instead of the lumen divide this number by two

and 2). PAX2p-null SCOUTs were then expressed per 10^5 microns (10 cm) of surface area (Table 2).

Statistical Analysis

Frequency data were analyzed with attention to both age and presence or absence of serous cancer. The data were analyzed to compare: the frequencies of PAX2p-null SCOUTs per tubal cross-section in cases (high-grade serous cancer) and controls (hysterectomies for benign ovarian disorders). The data were modeled on the assumption that the number of SCOUTs per unit surface length follows a Poisson distribution, with Poisson rate depending on group and age and extent of epithelial surface examined (as an offset). We further assumed that the Poisson rates are drawn from a gamma distribution in order to accommodate extra Poisson dispersion; this induces a negative binomial regression model. Statistical analysis was conducted in SAS, version 9.1 (Copyright (c) 2002–2003 by SAS Institute, Cary, NC, USA).

Results

Application of the Model for Precursor Frequency

Epithelial length was calculated in two ways. The first was by a single luminal measurement (Figures 2a and c) and the second by a linear measurement of both the luminal and basal epithelial interfaces (Figures 2b and d). In the latter calculation, the values were divided by two.

Frequency of Fallopian Tube Precursors

Table 2 expresses the frequency of SCOUTs as a function of 10^5 microns (10 cm perimeter length) examined. The number of PAX2p-null SCOUTs ranged from 0.00 to 4.60 for cases of serous cancer

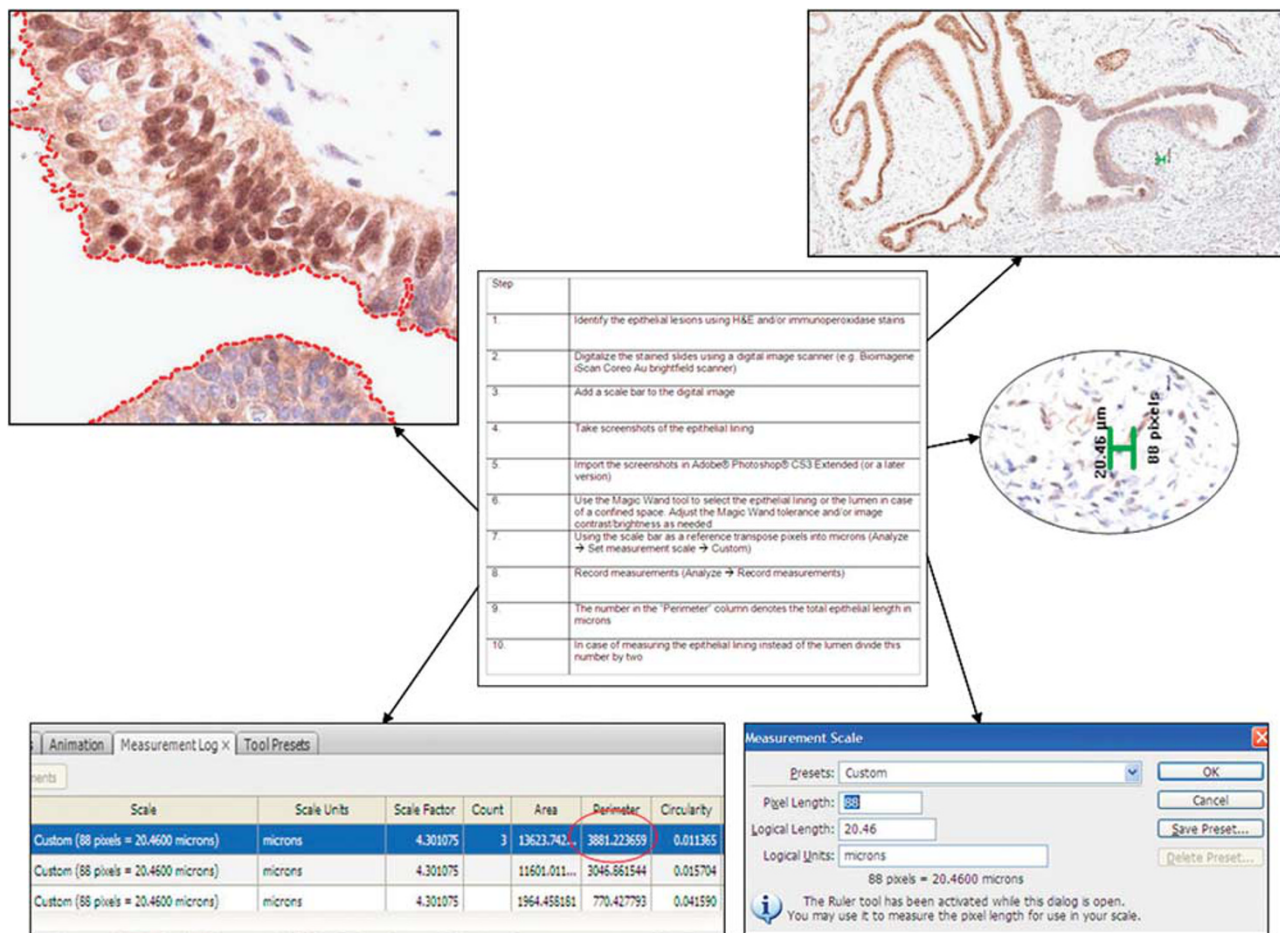


Figure 1 A 10-step model for measuring epithelial length in microns. The insets represent in a clockwise order starting at 12 o' clock: (1) immunoperoxidase stain (in this case PAX2) of an area of fallopian tube epithelium containing a SCOUT (PAX2p null area). (2) Digitally added scale bar converting pixels in microns. (3) Transposing measurements in Photoshop from pixels to microns. (4) Recorded measurements in Adobe Photoshop showing total epithelial length in microns. (5) Close up (60x magnification) of measurement with digitally enhanced measurement line.

and from 0.00 to 1.66 for controls, with means of 0.84 and 0.27, respectively, ($P = 0.007$, Student's *t*-test) (Figure 3). There was a significantly higher frequency of PAX2p-null SCOUTs in the serous cancer group by χ^2 analysis; overall cancers showed a 3.97-fold increase in SCOUT frequency compared with controls ($P < 0.0001$).

Required Perimeter Length for SCOUT Detection

Figure 4 illustrates the mean amount of epithelial length required in order to find from 1 to 3 SCOUTs. By measuring the epithelial length of all slides harboring a certain number of SCOUTs and combining these measurements, the mean epithelial length that is required to find one, two or three SCOUTs could be calculated. This presentation was devised to reduce the possibility of sampling bias introduced by the addition of one or multiple extra slides to a given case. These problems are eliminated using this representation because the total number of slides

per case is not taken into account, only the average amount of epithelium on slides that contain one or more SCOUTs. As can be seen the total length of epithelium that needs to be measured to find both one and two SCOUTs is significantly lower in serous cancer cases compared with controls. In order to find three SCOUTs, the epithelial length is still lower in serous cases, but the error bars overlap with the controls.

Topographic Distribution of SCOUTs

Figure 5 graphically depicts the physical distribution of SCOUTs on a slide, documenting whether multiple SCOUTs in a case tend to colocalize or have a random distribution throughout the fallopian tube. The frequency as plotted shows the pattern one would expect from a random distribution, with a high chance of having a single event in a given section; increasing numbers of events in a section become increasingly fewer in number.

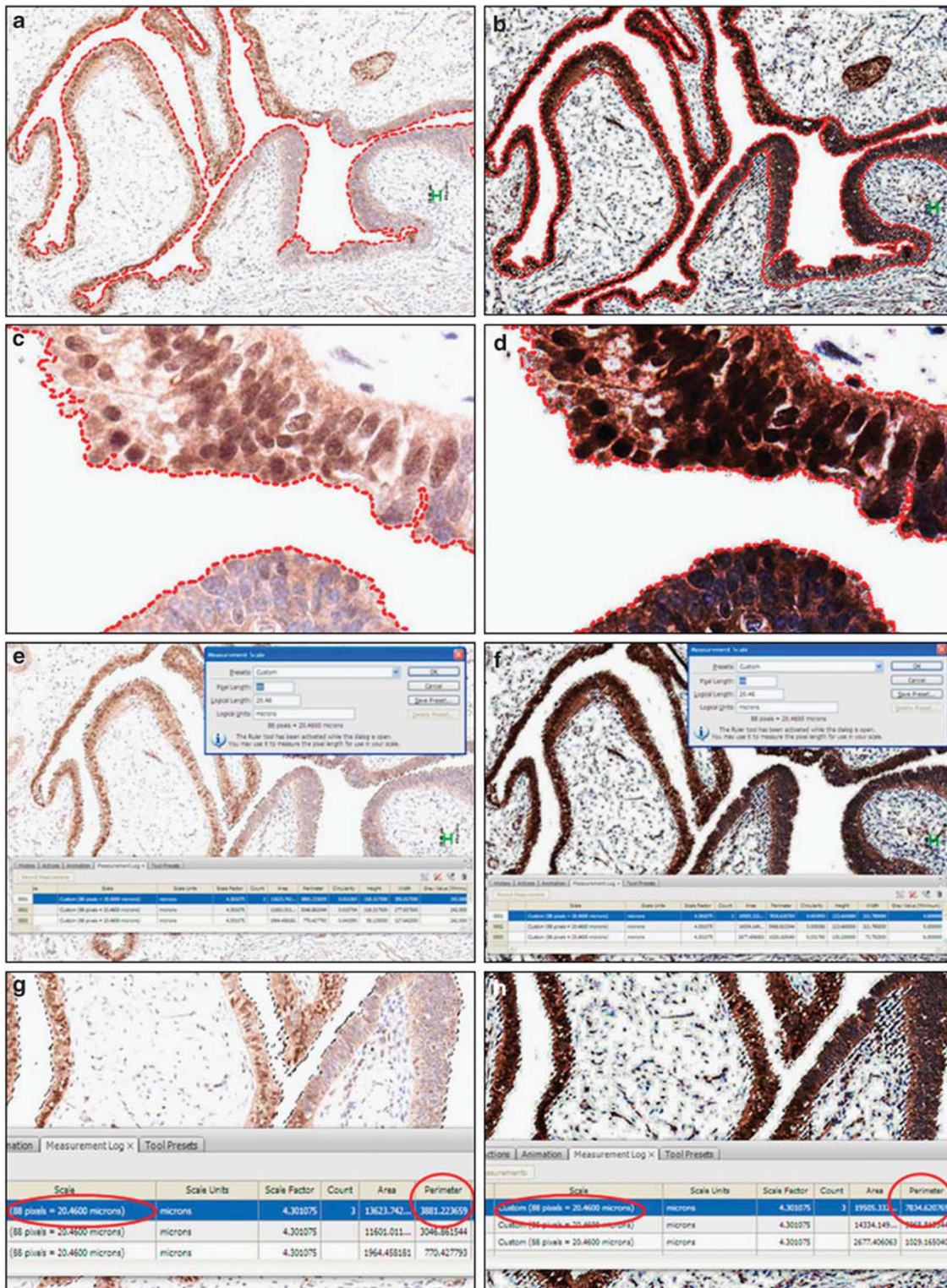


Figure 2 Epithelial measurement using both the lumen and epithelium measuring methods on the same tissue section stained for PAX2. (a) Measurement of the lumen with digitally enhanced measurement line. (b) Measurement of both the luminal and basal epithelial interfaces (contrast increased to enable measurement). (c, d) Close up at 60x magnification. (e, f) Real-time screenshot taken in Adobe Photoshop showing transposition of pixels in microns and recorded measurements. (g, h) Record of the measurements showing total epithelial length in microns in the 'Perimeter' column. When measuring the epithelium this number needs to be divided by two.

Table 2 Baseline table showing the age, SCOUT number, total epithelial length in microns and SCOUT/micron ratio for all cases and controls

Case	Pathology	Age	Epithelial length (micron)	SCOUT number	SCOUTs per 10 ⁵ microns (10 cm)
1	Serous OV CA	67	127 735.59	0	0
2	Serous OV CA	62	190 810.87	0	0
3	Serous OV CA	72	177 130.92	0	0
4	Serous OV CA	59	184 355.24	0	0
5	Serous OV CA	37	4 873 011.34	5	0.10
6	Serous OV CA	75	872 938.44	1	0.11
7	Serous OV CA	59	1 057 059.67	2	0.19
8	Serous OV CA	43	1 281 719.05	3	0.23
9	Serous OV CA	67	864 728.38	2	0.23
10	Serous OV CA	63	796 774.89	2	0.25
11	Serous OV CA	58	775 452.96	2	0.26
12	Serous OV CA	80	1 060 966.12	3	0.28
13	Serous OV CA	57	1 009 510.4	3	0.30
14	Serous OV CA	71	572 668.74	2	0.35
15	Serous OV CA	66	280 260.22	1	0.36
16	Serous OV CA	72	270 361.52	1	0.37
17	Serous OV CA	69	1 069 550.63	4	0.37
18	Serous OV CA	69	227 956.21	1	0.44
19	Serous OV CA	53	947 820.93	1	0.53
20	Serous OV CA	66	316 987.31	2	0.63
21	Serous OV CA	74	469 028	3	0.64
22	Serous OV CA	72	469 346.61	4	0.85
23	Serous OV CA	47	1 418 915.68	12	0.85
24	Serous OV CA	70	1 121 078.72	10	0.89
25	Serous OV CA	67	527 563.8	5	0.95
26	Serous OV CA	66	398 969.62	4	1.00
27	Serous OV CA	52	499 650.28	5	1.01
28	Serous OV CA	47	521 177.91	7	1.34
29	Serous OV CA	68	207 776.12	3	1.44
30	Serous OV CA	70	190 244.53	3	1.58
31	Serous OV CA	66	217 362.7	4	1.84
32	Serous OV CA	74	36 821.07	1	2.72
33	Serous OV CA	74	609 000.72	27	4.43
34	Serous OV CA	64	21 748.93	1	4.60
35	None	51	249 722.78	0	0
36	None	55	288 676.2	0	0
37	None	54	291 079.42	0	0
38	None	66	773 183.47	0	0
39	None	51	149 961.33	0	0
40	None	72	569 981.7	0	0
41	None	51	420 044.575	0	0
42	None	63	500 360.68	0	0
43	None	38	192 200.2	0	0
44	None	55	627 246.32	0	0
45	None	42	879 818.59	0	0
46	None	49	462 827.24	0	0
47	None	51	380 327.91	0	0
48	None	50	65 050.69	0	0
49	None	47	262 608.18	0	0
50	None	36	895 407.31	1	0.11
51	None	48	706 253.04	1	0.14
52	None	53	475 578.43	1	0.21
53	None	61	902 440.21	2	0.22
54	None	61	355 266.08	1	0.28
55	None	65	345 850.46	1	0.29
56	None	52	686 666.79	2	0.29
57	None	49	1 588 020.41	5	0.31
58	None	53	1 499 524.93	5	0.33
59	None	61	288 895.24	1	0.35
60	None	50	270 038.43	1	0.37
61	None	52	499 329.13	2	0.40
62	None	50	1 410 784.23	6	0.43
63	None	57	622 638.81	3	0.48
64	None	74	191 359.08	1	0.52
65	None	51	868 969.69	5	0.58

Table 2 Continued

Case	Pathology	Age	Epithelial length (micron)	SCOUT number	SCOUTs per 10 ⁵ microns (10 cm)
66	None	66	336 755.63	2	0.59
67	None	60	788 185.57	6	0.76
68	None	51	315 476.265	4	1.27
69	None	60	361 092.36	6	1.66

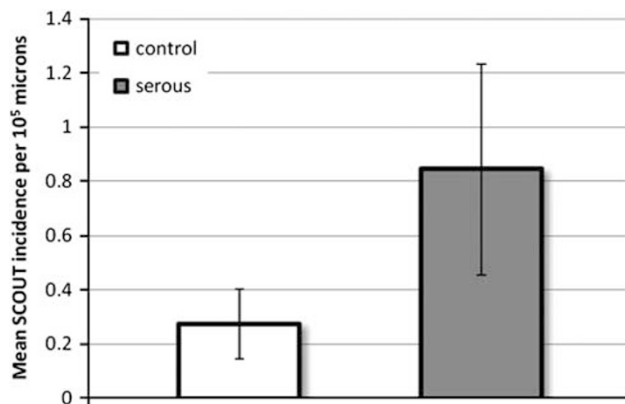


Figure 3 A graphic display of mean SCOUT incidence per 10⁵ microns, the error bars denote the 95% confidence interval.

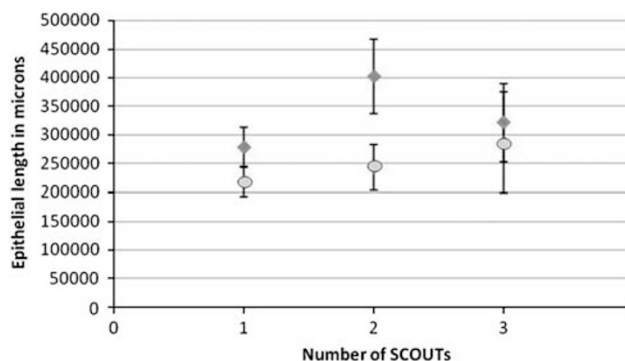


Figure 4 The mean epithelial perimeter length required to detect 1, 2 or 3 SCOUTs in cases and controls. Circles denote cancer-associated tubes and diamond shapes control tubes.

Influence of Age

The mean ages of serous cancer cases and controls were 64 (37–80) and 54 (36–74). Ages were categorized into quartiles of its distribution (<51, 51–60, 60–67 and >67) and found to be significantly associated with number of SCOUTs in this model ($P=0.025$). However, serous cancers and controls remained significantly different on adjusting for age in the negative binomial regression model ($P=0.001$), with cancers having approximately 4.1 times as many SCOUTs as controls.

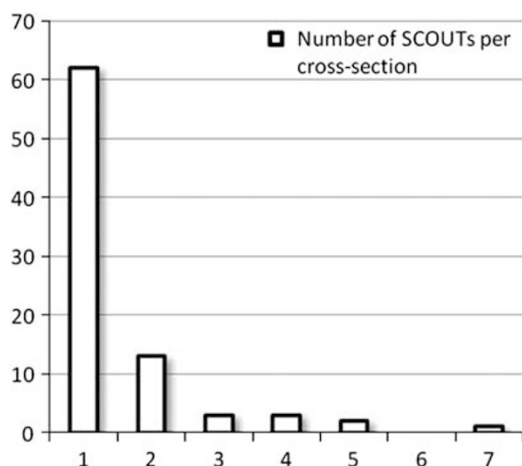


Figure 5 A graphic representation of SCOUTs per section. In most tissue sections, a single SCOUT is observed, with progressively fewer sections exhibiting two or more SCOUTs. This supports the concept that SCOUTs do not tend to cluster in specific loci (as represented by tissue sections).

Discussion

This paper illustrates a method by which an objective assessment of fallopian tubeal mucosal length can be achieved for the purposes of fixing a denominator to the number of precursors tabulated in a histologic specimen in two dimensions. If tabulated in serial sections, a reproducible three-dimensional estimate would also be possible. The implications of this approach are considerable given the differences observed in fallopian tube precursor frequency and the potential link between precursor frequency and cancer risk in the fallopian tube and other organs. The p53 signature is considered a putative direct precursor to serous tubal intraepithelial carcinoma and initial estimates did not detect a significantly higher frequency of p53 signatures in tubes from women with *BRCA1* or *BRCA2* mutations relative to controls.^{9,25} Others observed a higher frequency in tubes from women at genetic risk or in older women.^{4,26} However, estimates could be influenced by the number of tissue blocks or the number of serial sections examined.¹⁴

SCOUTs have emerged as a form of 'surrogate precursor', that is, they are more widely distributed in the fallopian tube than p53 signatures and lack *TP53* mutations, but share functional gene disturbance with malignancies (loss of PAX2p expression) and appear to be more frequent in women with serous cancer. Thus, SCOUTs may be a harbinger of one or more biologic events that predate and signal an increased risk of neoplasia. SCOUTs were calculated as nearly sixfold more frequent in cancer cases when compared with controls.¹³ Follow-up studies recognized the need to account for both the age of the subjects and number of cross-sections examined.^{12,13} When this was done, an additional link to age and a less pronounced (albeit still significant) relationship to malignancy was observed. This study

was designed to more critically evaluate this relationship and create a method by which the surface area of the fallopian tube could be ascertained. As shown in Figures 1 and 2, the assessment of epithelial surface is easily accomplished by scanning the slide and digitizing the images with scale bars followed by an analysis in Adobe Photoshop to compute the linear surface in microns. With this approach, the proposed link between SCOUT frequency (per 10^5 microns or 10 centimeters) and neoplasia is validated.^{11–13} We found in addition, as would be expected, that the mean amount of perimeter needed to identify the first SCOUT was less in cases of serous cancer. Moreover, the distribution of SCOUTs appeared to be random, without clustering in particular tissue sections of the fallopian tube (Figure 5).

Morphologically benign epithelial lesions similar to SCOUTs have been shown to be an early step in carcinogenesis in several other tissues, including the colon, prostate and pancreas.^{27–29} In the latter, the putative precursor is designated pancreatic intraepithelial neoplasia or PanIN. PanIN has been defined as a microscopic lesion arising in a smaller (<0.5 cm) pancreatic duct.³⁰ Morphologically benign flat and papillary pancreatic precursors (PanIN-1A and 1B) contrast with severe atypias (PanIN-3), similar to SCOUTs and p53 signatures and tubal intraepithelial carcinomas of the fallopian tube.³¹ Like SCOUTs and p53 signatures, low-grade PanINs are common in the general population but are more common in cancers *vs* controls (43% *vs* 28%).³² Like tubal intraepithelial carcinoma, high-grade PanIN is much rarer and even more likely to be associated with malignancy.³³ Overall, PanINs increase in frequency as a function of age, similar to SCOUTs and (in more recent reports) p53 signatures.^{4,12,13,26,31,34,35}

Currently used methods of assessing PanIN frequency are by either measuring the number of PanINs per cm^2 of pancreatic tissue^{19,20,36} or by measuring PanIN frequency per slide.³² The first method measures both epithelium and parenchyme within the studied fields and as such measuring errors may occur when comparing the frequency of PanINs in fields with a variable epithelial/parenchymal ratio. The second method could induce sampling bias because of a variable number of reviewed slides between patients. This concern was previously expressed by Andea *et al*³² and in their study they corrected for the presence of sampling bias by adding the number of slides as an independent variable to a multivariate analysis. The method described in this study, measuring frequency as a function of total epithelial length, is more precise and might be useful in this system as well. Given the emerging link between early mucosal events and cancer risk in this and other reports, ascertainment of precursor frequency will have a critical major role in both sub-classifying populations of women with epithelial cancer and ascertaining the efficacy of preventive strategies.^{27,29}

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Author contributions

JGB—performed digital analyses, performed immunohistochemistry, wrote and edited the manuscript. GN—performed immunohistochemistry and reviewed the manuscript. ARL—reviewed the pathology, scored stains and reviewed the manuscript. CMQ—reviewed the pathology, scored stains and reviewed the manuscript. RAB—performed statistical analyses and reviewed the manuscript. NMM—devised the digital approach with JGB, scanned cases and reviewed the manuscript. EK—devised statistical approaches and reviewed the manuscript. FDM—devised study, supervised GN and reviewed the manuscript. WX—devised study, supervised GN and reviewed the manuscript. CPC—devised study, wrote and reviewed the manuscript.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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